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Adenylosuccinate Lyase from *Artemia* Embryos

PURIFICATION AND PROPERTIES*

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In crude extracts, the molecular form of adenylosuccinate lyase is pH-dependent as studied by gel filtration and sucrose gradient centrifugation. At pH values of 8.7 and 6.5, the enzyme exhibits molecular forms of 200 kDa and larger than 500 kDa, respectively. At pH values of 7.0–7.5, forms of intermediate molecular weight were detected. Interconversion among the different molecular forms of adenylosuccinate lyase was not achieved when the enzyme was subjected to two successive chromatographic steps on Sepharose CL-6B using elution buffers at two different pH values. A unique form of 200 kDa was observed, regardless of the pH of the buffer used, upon either gel filtration or sucrose gradient centrifugation in the presence of 1 M NaCl.

The enzyme from *Artemia* cysts was purified to homogeneity. It had molecular mass of 200 kDa and gave a single band of 56 kDa upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The K_m values for adenylosuccinate, AMP, and fumarate were 1, 36, and 350 μM, respectively. The enzyme exhibits a Uni Bi-ordered mechanism and is maximally active at a pH value of 8.0–8.5. For maximum activity, the enzyme requires an ionic strength equivalent to 80 mM KCl. The isoelectric point determined by chromatofocusing was 5.04.

During development from encysted gastrula to free swimming larva, *Artemia* uses stored diguanosine tetraphosphate as a source of both adenine and guanine nucleotides (1). An investigation of this metabolic pathway led us to the study of diguanosine tetraphosphatase (EC 3.6.1.17) (2), GMP reductase (EC 1.6.6.8) (3), and adenylosuccinate synthetase (EC 6.3.4.4) (4) in *Artemia* cysts extracts. These three enzymes acting in sequence generate adenylosuccinate from the GMP moiety of diguanosine tetraphosphate. In this paper we describe the presence and molecular and kinetic properties of adenylosuccinate lyase (EC 4.3.2.2) from *Artemia*. This enzyme splits adenylosuccinate into fumarate and AMP and represents the last step in the synthesis of this adenine nucleotide from diguanosine tetraphosphate.

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EXPERIMENTAL PROCEDURES

Materials

Artemia cysts were obtained from Bio-Marine Research, Hawthorne, CA. Sepharose CL-6B, Sepharose 4B, Sephacryl S-300 (superfine), Sephadex G-25M, Polybuffer exchanger PBE 94, Polybuffer 74, and the electrophoresis calibration kit were obtained from Pharmacia Fine Chemicals. DEAE-cellulose (0.85 meq/g) was obtained from Serva and Cibacron blue F3G-A from Ciba AG, Basel. Adenylosuccinate was purchased from Sigma and AMP and fumarate from Boehringer Mannheim. Proteins used as molecular weight markers in gel filtration and sucrose gradient centrifugation were from Boehringer Mannheim. Materials used for polyacrylamide gel electrophoresis were obtained from Merck.

Methods

Preparation of the Extracts—Before use, the cysts were hydrated overnight in glass-distilled water (20 ml/g of cysts). The cysts that sedimented were collected and washed twice with glass-distilled water, followed by treatment with 1% cold NaClO for 15 min. This chemical was removed by successive washes in cold distilled water. A last wash was performed with homogenization buffer, and the cysts were filtered through a piece of cloth. These operations were carried out near 4 °C.

The wet cysts were disrupted in a mortar. After proper homogenization was accomplished, as checked by phase-contrast microscopy, approximately 1.5 volumes of buffer were added per g of the sample, original dry weight, and the resulting homogenate was filtered through glass wool and centrifuged at 700 × *g* for 5 min. The supernatant was again filtered through glass wool and centrifuged at 27,000 × *g* for 2 h.

Adenylosuccinate Lyase (EC 4.3.2.2) Assay—The K_{eq} value (6.8×10^{-3} M) of the reaction catalyzed by this enzyme (5) allows one to measure the enzymatic activity in both directions. In the forward direction, the assay mixture contained, in a final volume of 1 ml, the following components: 50 mM Tris-HCl buffer, pH 7.5, 0.025 mM EDTA, 0.015 mM adenylosuccinate and enzyme. A control without substrate was carried out in parallel. The reaction was initiated by the addition of the substrate and was followed at 280 nm in 1-cm light path cuvettes maintained at 30 °C. The transformation of 1 μmol of adenylosuccinate into AMP and fumarate is associated with a decrease of 10.7 A_{280} units (5). In the reverse direction, the assay mixture contained, in a final volume of 1 ml, the following components: 50 mM sodium phosphate buffer, pH 6.5, 0.2 mM AMP, 2.25 mM fumarate and enzyme. A control without AMP was run in parallel. The reaction was initiated by the addition of fumarate and followed at 280 nm.

Throughout this work, adenylosuccinate lyase activity was determined in the forward direction, except when indicated. One unit of adenylosuccinate lyase is the amount of enzyme able to transform 1 μmol of substrate/min in the above conditions.

Catalase (EC 1.11.1.6) Assay—The reaction mixture contained the following components in a final volume of 1 ml: 50 mM sodium phosphate buffer, pH 7.0, 10 mM H_2O_2 and enzyme. Decrease in absorbance was followed at 240 nm. H_2O_2 was prepared shortly before use as a 0.3 M solution in 0.1 M sodium phosphate buffer, pH 7.0, and 0.05 mM EDTA. This assay was performed at room temperature.

Other enzyme activities were determined as previously described (4).

SDS[1]*-Polyacrylamide Gel Electrophoresis*—Discontinuous SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli (6). The gels were 10 cm long and 0.5 mm thick, with 2.9% acrylamide stacking gel containing 125 mM Tris-HCl, pH 6.8, 0.1% methylenebisacrylamide, 0.1% SDS and a 8.7% acrylamide running gel containing 375 mM Tris-HCl, pH 8.8, 0.3% methylenebisacrylamide, and 0.1% SDS. The samples were prepared in a volume of 100 μl of 62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.005% bromphenol blue. This mixture was then heated at 100 °C for 5 min and cooled on ice. Molecular markers used were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). A constant current of 3 mA was applied until the dye entered the running gel. The current was then increased to 15 mA and continued at this level until the marker dye front was 1 cm from the front of the gel. Electrophoresis was performed in the cold room. After completion of the run, the gels were stained and destained as described by Weber and Osborn (7).

Coupling of Cibacron Blue F3G-A to Sepharose 4B—This coupling was performed essentially as described by Böhme *et al.* (8). A solution of 1 g of Cibacron F3G-A in 30 ml of glass-distilled water was added dropwise with vigorous stirring to a suspension of 100 ml of Sepharose 4B diluted to 1 liter with glass-distilled water at a temperature of 60 °C. After stirring for 15 min longer, once dye addition was completed, 45 g of sodium chloride were added and the stirring was continued for 30 min. After that, the mixture was heated to 71 °C, treated with 4 g of Na_2CO_3, and kept for a further 1.5 h at this temperature with gentle stirring. After cooling to room temperature, the gel was filtered by suction on a Buchner funnel and washed with glass-distilled water until the filtrate became colorless.

Protein Assay—Protein concentrations were determined by the method of Lowry *et al.* (9), except for purified enzyme preparations from steps 4 and 5 where they were measured by UV absorption (10).

The pH of all buffers used for homogenization and column elutions was adjusted at 4 °C, the standard temperature for all experimental conditions.

RESULTS

Molecular Forms

Initial experiments conducted to establish the best conditions for the purification of the enzyme showed that the chromatographic behavior on Sephadex G-200 of adenylosuccinate lyase present in the 27,000 × *g* supernatant differed when two different elution buffers were used. As shown below, a systematic search for the reason for that phenomenon pointed to the pH value of the elution buffer as the main cause of the changing chromatographic position of the enzyme (Fig. 1). Shortly before use, a 27,000 × *g* supernatant was prepared as described under "Methods" using 20 mM Tris-HCl, 0.5 mM EDTA (buffer A), pH 7.5, as homogenization buffer. Aliquots of 3 ml of this supernatant were applied successively to a Sepharose CL-6B column (1.6 × 66 cm) previously equilibrated in buffer A adjusted at pH 6.5, 7.0, 7.5, and 8.7, and the sample eluted with the same equilibrating buffer. Additionally the column was calibrated with the following markers: ferritin, catalase, bovine serum albumin, and calf intestine adenosine deaminase. Each marker was applied separately, dissolved in 3 ml of buffer A, pH 7.5, and eluted with the same buffer. From Fig. 1 it is clear that at pH 8.7 and 6.5, the enzyme eluted at positions corresponding to molecular weights of 2×10^5 and larger than 5×10^5, respectively. A drastic change in the molecular weight occurs between pH 7.0 and 7.5. In addition, the presence of forms with intermediate molecular weights can be inferred from the elution profile of the enzyme at those two pH values.

The possible interconversion among the different molecular forms of adenylosuccinate lyase was studied by subjecting the enzyme to two successive chromatographic steps using elution buffers at two different pH values. Three aliquots of an ammonium sulfate fraction (40–50%) of a 27,000 × *g* supernatant of *Artemia* cysts obtained in buffer A, pH 7.5, were separately chromatographed on Sepharose CL-6B using as eluting agent buffer A adjusted at three different pH values: 8.7, 7.1, and 7.5 (Fig. 2). The fractions with maximal activities were pooled and re-chromatographed on the same Sepharose CL-6B column equilibrated and eluted with buffer A at pH values of 6.6, 8.7, and 7.5, respectively. The results presented in Fig. 2 show that, as expected, adenylosuccinate lyase eluted from the first chromatographic run in two different forms, larger and smaller, depending on the pH (7.1 and 8.7, respectively), of the elution buffer. At pH 7.5, the presence of the two forms is apparent. Unexpectedly, however, in the second chromatographic run only the smaller form of the enzyme, corresponding to a molecular weight of around 200,000, can be isolated even at acidic (6.6) pH. It is also worthy of note that rechromatography of the enzyme at pH 7.5 causes the disappearance of the form of larger molecular weight.

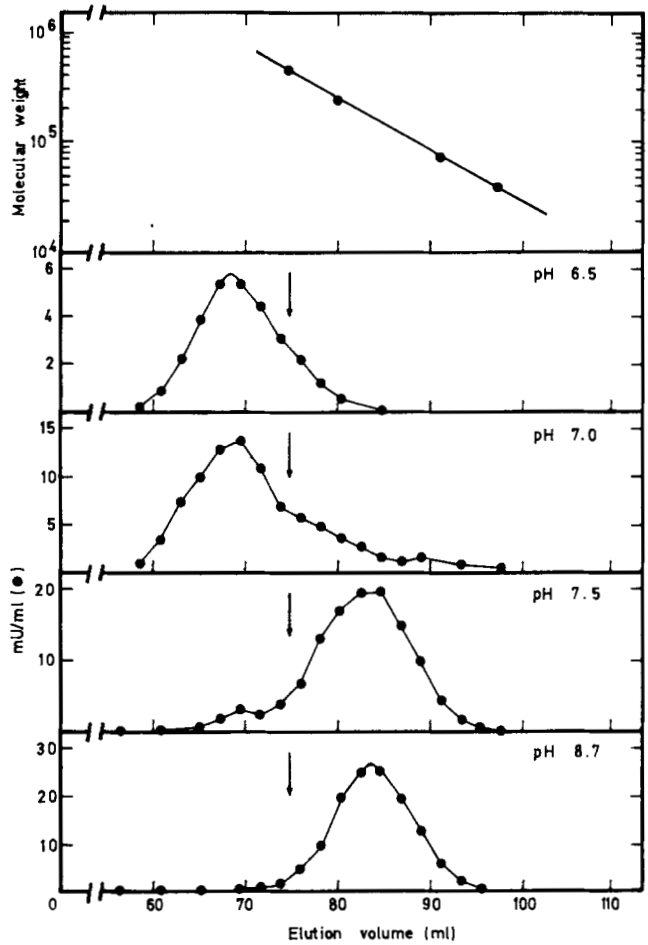

FIG. 1. **Chromatography on Sepharose CL-6B of adenylosuccinate lyase from a 27,000 × *g* supernatant of *Artemia* cysts.** The size of the column was 1.6 × 66 cm. Fractions were collected every 7.5 min and their volume determined by weight. Flow rate was 18 ml/h. The column was calibrated with markers of known molecular weight (ferritin, 450,000; catalase 240,000; bovine serum albumin, 68,000; and calf intestine adenosine deaminase, 38,000) applied separately and dissolved in 3 ml of 20 mM Tris-HCl, 0.5 mM EDTA (buffer A), pH 7.5 (top panel). In the other panels, 3-ml aliquots (≃55 mg of protein) of a 27,000 × *g Artemia* cyst supernatant, obtained in buffer A, pH 7.5, were applied to different columns equilibrated and eluted with buffer A adjusted to the pH indicated in the figure. For simplicity, the elution profile of the 27,000 × *g* supernatant protein is not represented; in all cases, maximum absorption at 280 nm was obtained in fraction 50.

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

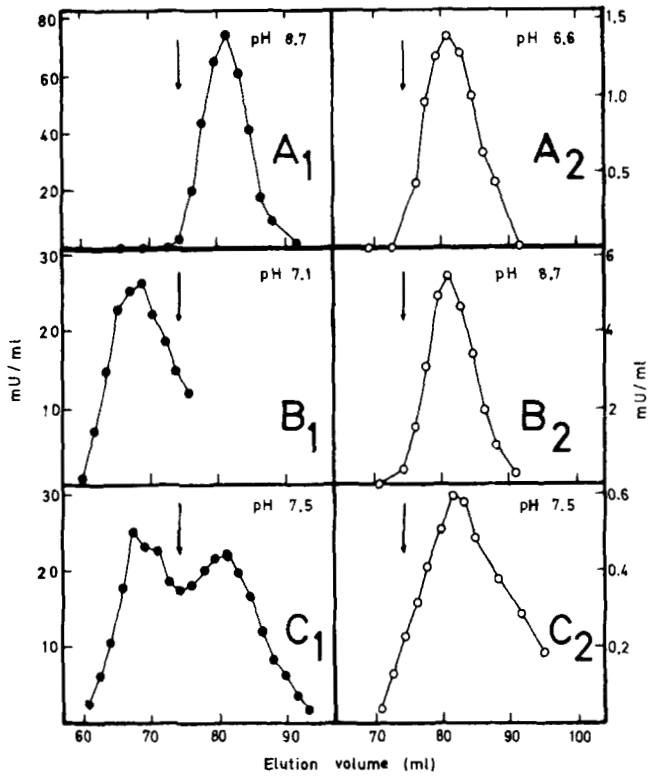

FIG. 2. **Chromatography and re-chromatography on Sepharose CL-6B of adenylosuccinate lyase present in the $(NH_4)_2SO_4$ (40–50%) of *Artemia* cyst extracts.** A 27,000 × *g* supernatant was obtained from *Artemia* cysts homogenized in 5 volumes of buffer A, pH 7.5/g of cysts, dry weight. 50-ml aliquots of the supernatant were fractionated with $(NH_4)_2SO_4$ between 40 and 50 saturation. The precipitate was resuspended in 4.0 ml of buffer A adjusted at pH values of 8.7, 7.1, and 7.5. Aliquots of 3 ml (containing around 90 mg of protein) were applied to a Sepharose CL-6B column (1.6 × 66 cm) equilibrated and eluted with the same buffer in which the precipitate was resuspended. Fractions containing the major portion of adenylosuccinate lyase activity were pooled, and 3-ml aliquots (containing around 3 mg of protein) were re-chromatographed on the same column, previously equilibrated in buffer A adjusted at the pH indicated in the figure and eluted with the same buffer. Other experimental conditions were as described in the legend to Fig. 1. A_1, B_1, and C_1 and A_2, B_2, and C_2 denote the first and second chromatography, respectively, carried out at the pH indicated. *Arrow* indicates the elution position of ferritin.

Ionic strength also influences the size of the enzyme. In an experiment similar to that described in the legend to Fig. 1, the 27,000 × *g* supernatant was allowed to stand for 2 h at pH 7.5 and 2 °C in the presence of 1 M NaCl and then centrifuged at 27,000 × *g* for 15 min. All the enzymatic activity was recovered in the supernatant after this treatment. An aliquot of 3 ml of the supernatant was applied to a Sepharose CL-6B column equilibrated in 1 M NaCl in buffer A, pH 7.5. The enzyme eluted as a single peak. Its molecular weight, determined with the help of markers of known molecular weight eluted with the same buffer, was 200,000 (data not shown).

Similar results on the molecular forms of the enzyme in relation to pH were obtained using sucrose gradient centrifugation. Supernatants (27,000 × *g*) of *Artemia* were obtained from 3 g of cysts homogenized in 3 volumes of buffer A, adjusted at pH values of 7.0, 7.6, and 8.6. In all cases, the supernatants contained, in a volume of approximately 10 ml, 445 milliunits of enzyme and 160 mg of protein. Aliquots were layered on the top of a 10-ml sucrose gradient (5–20%) prepared in the same buffer A in which the supernatants were obtained. After centrifugation at 38,000 rpm for 14 h using an SW 41 rotor in a Beckman Model L5-65 ultracentrifuge, fractions were collected from the bottom of the gradients and the enzyme activity was measured. The total enzymatic activity recovered from the gradients was about 80% of that applied. At a pH value of 7.0, 60% of the enzyme sedimented as a molecular form larger than 500,000 and 40% as a molecular form of 200,000; at a pH value of 8.6, only the molecular form of 200,000 was recovered and at the intermediate pH value of 7.6, besides the molecular form of 200,000, 30% of activity was recovered with M_r = 300,000. Molecular weights were determined by the method of Martin and Ames (11) (Fig. 3). When the pH value of the supernatant obtained at pH 8.6 was lowered to 6.8 with HCl and the mixture was centrifuged in a sucrose gradient in buffer A at pH 6.8, 54% of the recovered enzymatic activity was larger than M_r = 500,000.

Using the same experimental conditions, but with 1 M NaCl or 1 mM *p*-hydroximercuribenzoate supplementing both the 27,000 × *g* supernatant and the sucrose gradients, an unique enzymatic form of M_r = 200,000 was obtained when the

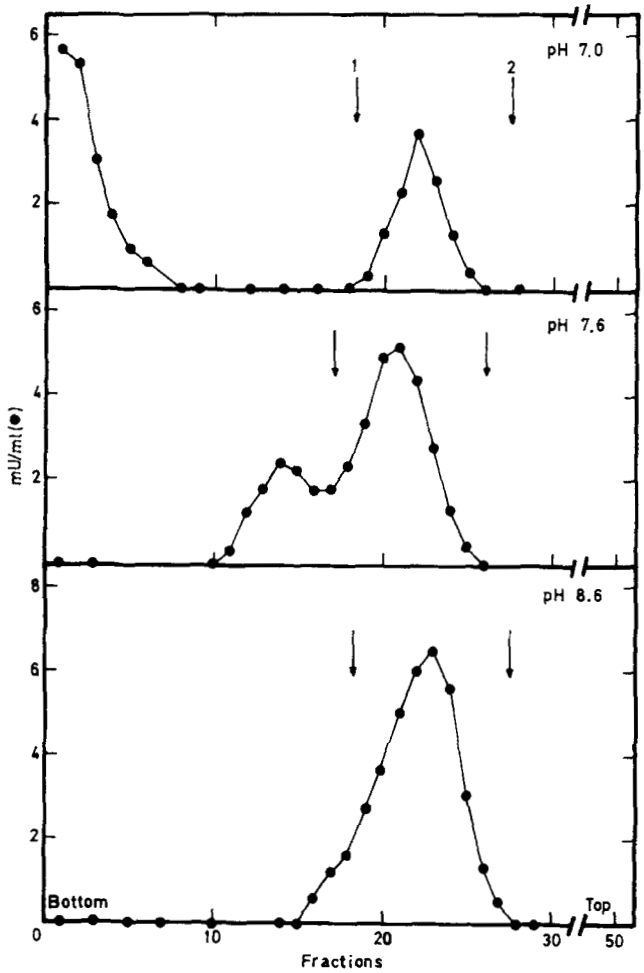

FIG. 3. **Centrifugation of adenylosuccinate lyase in sucrose gradients.** 27,000 × *g* supernatants from *Artemia* cysts were obtained in buffer A, equilibrated at pH values of 7.0, 7.6, and 8.6. Samples of 200 μl (containing 190 μl of supernatant, 5 μl of catalase (8 mg/ml), and 5 μl of alcohol dehydrogenase (4 mg/ml)) were loaded on a 10-ml sucrose gradient (5–20%) in the same buffer in which the 27,000 × *g* supernatants were obtained. The gradients were centrifuged at 4 °C for 14 h at 38,000 rpm in a Beckman SW 41 rotor. Fractions of 0.2 ml were collected from the bottom of the gradient. Catalase and alcohol dehydrogenase sedimented in the positions indicated by *arrows 1* and *2*, respectively.

centrifugations were carried out in a medium at pH 7.0 (data not shown).

Purification of the Enzyme

The experimental conditions for the purification of adenylosuccinate lyase were chosen to favor the occurrence of the enzyme in the M_r = 200,000 form. All operations were carried out at 4 °C.

Step 1: 27,000 × g Supernatant—40 g of *Artemia* cysts treated as described under "Methods" were homogenized in 250 ml buffer A, pH 7.5; the brei was centrifuged for 5 min at 700 × *g*, and the supernatant was centrifuged again for 2 h at 27,000 × *g*. A supernatant with a volume of 250 ml was obtained.

Step 2: Ammonium Sulfate Fractionation—To the solution from the previous step, 24.3 g of solid ammonium sulfate/100 ml (0.4 saturation) were added. After stirring for 30 min, the suspension was centrifuged at 27,000 × *g* for 15 min and the precipitate was discarded. The supernatant was brought to 0.5 saturation with ammonium sulfate by the addition of 6.3 g/100 ml and treated as above. The precipitate was resuspended in 10.5 ml of buffer A, pH 7.5, supplemented with 0.3 M KCl.

Step 3: Chromatography on Sephacryl S-300—The solution from the previous step was kept for 2 h at 4 °C and applied to a Sephacryl S-300 column (2.6 × 65 cm) equilibrated with 0.3 M KCl in buffer A, pH 7.5. The material was eluted with the same buffer at a flow rate of 30 ml/h. Fractions of 3 ml were collected. The enzyme eluted as a single, symmetric peak. The major portion of the activity was in fractions 54–64, in a volume of 36.7 (data not shown).

Step 4: Chromatography on DEAE-cellulose—To 17 ml of enzyme solution obtained in the previous step, an equal volume of buffer A, pH 7.5, was added to bring the KCl concentration to 0.15 M. The solution was applied to a DEAE-cellulose column (1.9 × 7 cm) equilibrated with buffer A, pH 7.5, supplemented with 0.15 M KCl (Fig. 4). The column was then washed with the same buffer until the absorbance at 280 nm of the effluent was near zero. Adenylosuccinate lyase was eluted with 100 ml of a linear gradient (0.15–0.5 M) of KCl in buffer A, pH 7.5. The volume of the fractions was of 3 ml and the flow rate 15 ml/h. Fractions 87–118 were pooled and processed as follows.

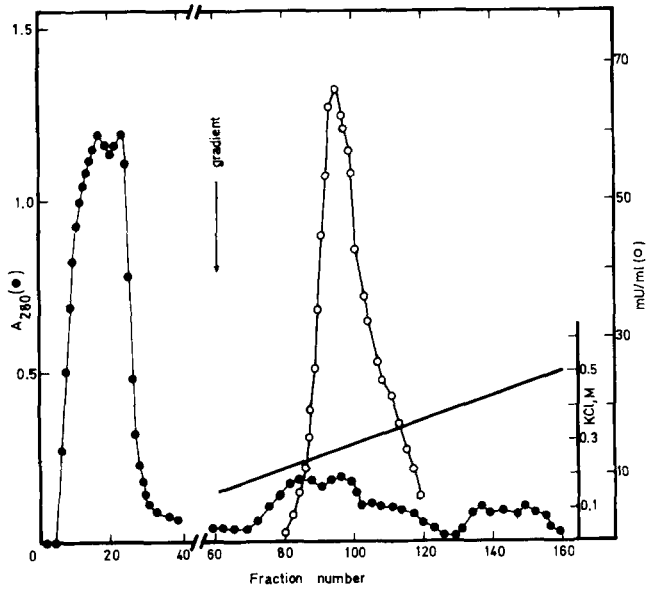

FIG. 4. **Chromatography of adenylosuccinate lyase on DEAE-cellulose.** 102 mg of protein from step 3, in buffer A, pH 7.5, 0.15 M KCl, were applied to a DEAE-cellulose column (1.9 × 7 cm) equilibrated in the same buffer. The column was washed with the same buffer, and the enzyme was eluted with 100 ml of a linear KCl gradient (0.15–0.5 M) in buffer A, pH 7.5.

Step 5: Affinity Chromatography on Cibacron Blue F3G-A-Sepharose 4B—40 ml (6.4 mg of protein) of the above pool were brought to 440 ml with buffer A, pH 7.5, and applied to a Cibacron blue-Sepharose column (2.1 × 3.2 cm) previously equilibrated in buffer A, pH 7.5. The column was then washed with 30 ml of the same buffer. Adenylosuccinate lyase activity was eluted with 50 ml of a linear KCl gradient (0–0.4 M) in buffer A, pH 7.5, and 2-ml fractions were collected at a flow rate of 19 ml/h. Fractions 5–12 comprising the major portion of the enzymatic activity were pooled. A summary of a typical purification run is presented in Table I. A purification of 800-fold with a recovery of 46% was obtained with this procedure.

Molecular Weight Determination in the Absence and Presence of Sodium Dodecyl Sulfate

The molecular weight of enzyme from step 4 was determined by sucrose gradient density centrifugation at pH 7.4, as described above. The molecular mass determined for adenylosuccinate lyase was 187 or 220 kDa using catalase or alcohol dehydrogenase as reference proteins, respectively. Two peaks of enzymatic activity were obtained when a 27,000 × *g* supernatant was centrifuged in parallel in the same experimental conditions: one with the same molecular weight as the purified enzyme (53% of the recovered activity) and a second one, at the bottom of the gradient, corresponding to a molecular mass larger than 500 kDa.

To estimate the subunit molecular weight of purified enzyme from step 5, polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (6) on a 7.5% polyacrylamide slab. The enzyme was first precipitated with 10% (final concentration) trichloroacetic acid for 3 h at 2 °C. The precipitate was washed twice with ethanol:ether (1:1) and once with ether, dissolved in sample buffer, and subjected to electrophoresis. A main band with M_r = 55,000 and two faint bands with M_r = 45,000 and 40,000 were observed.

In another experiment, fractions with maximal enzyme activity from step 5 of the purification were pooled, desalted, and re-chromatographed on a Cibacron blue-Sepharose column using the same experimental conditions as previously described. Fractions with maximal enzyme activity and undetectable protein by UV absorption were pooled, concentrated by placing 4-ml aliquots in dialysis tubing, and immersed in solid sucrose until the volume of the solution decreased to 200 μl. These solutions were pooled and dialyzed against sample buffer (see "Methods"). Aliquots of 100 μl (containing ≃1 μg of protein) were subjected to SDS-polyac-

TABLE I

Purification of adenylosuccinate lyase from Artemia embryos

Step	Volume	Protein	Activity	Specific activity	Yield
	ml	*mg*	*units*	*milliunits/mg*	%
1. 27,000 × *g* supernatant	250	2,500	7.3	2.9	100
2. $(NH_4)_2SO_4$ fractionation	10.5	630	5.2	8.3	72
3. Sephacryl S-300 chromatography	36.7	220	5.0	22.5	68
4. DEAE-cellulose chromatography	138.3	22	4.5	204.3	62
5. Cibacron blue-Sepharose chromatography	55.7	1.4	3.3	2,400	46

rylamide gel electrophoresis as described under "Methods." Under these conditions, an unique band was observed with a $M_r = 56{,}000$.

Kinetic Properties of the Enzyme

The velocity of the reaction was measured at different concentrations of adenylosuccinate. The substrate concentration activity curve was hyperbolic and yielded an apparent Michaelis constant of around 1.2 μM. The effect of two concentrations (50–75 μM) of AMP was assayed in the presence of adenylosuccinate in a range of concentrations between 2 and 10 μM. AMP was a competitive inhibitor of the reaction (Fig. 5). From a representation of the slopes of the Lineweaver-Burk plot (12) *versus* AMP concentration, a straight line was obtained and a K_i value of 18 μM was determined for AMP. Fumarate was a noncompetitive inhibitor (Fig. 6) as shown by a double reciprocal plot obtained in the presence of variable concentrations of substrate and fixed concentrations (0.45, 0.9, and 1.8 mM) of fumarate. Replots (Fig. 6, *inset*) of the slopes or intercepts *versus* fumarate concentrations gave two straight lines from which $K_{i_{slope}}$ and $K_{i_{intercept}}$ values of 1.9 and 1.15 mM were obtained. As with the enzyme from other sources (13, 14), the product inhibition data are consistent with an ordered Uni Bi kinetic mechanism model in which fumarate does not combine with the free enzyme to an appreciable extent.

The reaction can also be followed in the reverse direction. In that case, a K_m value for AMP (36 μM) was obtained in the presence of a saturating concentration of fumarate (4.5 mM) and variable concentrations (20–50 μM) of AMP. In the same way, a K_m value for fumarate (0.35 mM) was calculated in the presence of variable concentrations (0.18–1.1 mM) of this substrate and a saturating concentration of AMP (0.5 mM) (data not shown).

Other Properties of the Enzyme

Adenylosuccinate lyase requires a certain ionic strength for maximum activity as reported for the enzyme from other sources (15). When in the usual assay conditions for the forward reaction, the Tris-HCl concentration was reduced to 0.6 mM, the observed activity was practically negligible. Under these conditions, addition of increasing concentrations of KCl, up to a maximum of 80 mM, stimulated the enzymatic activity. Higher concentrations of KCl were inhibitory. At 200 mM KCl, only 50% of the maximum activity was attained. Addition of KCl to the usual reaction mixture (50 mM Tris-HCl, pH 7.5) inhibited the enzymatic activity; in the presence of 80 and 200 mM KCl, only 50 and 10%, respectively, of the control activity were measured.

The effect of pH on the activity of the enzyme was evaluated over the pH range of 6–9.5 using 50 mM Tris-HCl as buffer. The enzyme exhibited maximum activity at the pH range 8.0–8.5, and it was inactive at pH values below 6 and over 9.5.

The isoelectric point of the enzyme was determined by chromatofocusing as follows. An aliquot of 18 ml of enzyme preparation from step 4 of the purification was brought to 63 ml with buffer A, pH 7.5, and applied to a DEAE-cellulose column of 2 ml, previously equilibrated in the same buffer. The enzymatic activity was eluted in a small volume (5 ml) with 0.5 M KCl in buffer A, pH 7.5. Aliquots of 2.5 ml of this preparation were separately desalted using a Sephadex G-25M column (1 × 5 cm), previously equilibrated with 25 mM imidazole-HCl, pH 7.4, and eluted with the same buffer. With this procedure, the original enzyme preparation was concentrated from 18 to 7 ml and changed to a convenient buffer. In order to protect the enzyme, enough solid bovine serum albumin was then added to the sample to bring the solution to 10 mg of albumin/ml. The isoelectric point was determined with a column of Polybuffer exchanger PBE 94 (0.9 × 23 cm), following the instructions contained in the handbook provided by Pharmacia Fine Chemicals. The column was first equilibrated with degassed 25 mM imidazole-HCl buffer, pH 7.4, and then washed with 5 ml of Polybuffer 74 (diluted 9-fold with glass-distilled water, adjusted to pH 4 with HCl, and degassed). The sample (7 ml), prepared as described above, was applied to the column and eluted with 210 ml of the

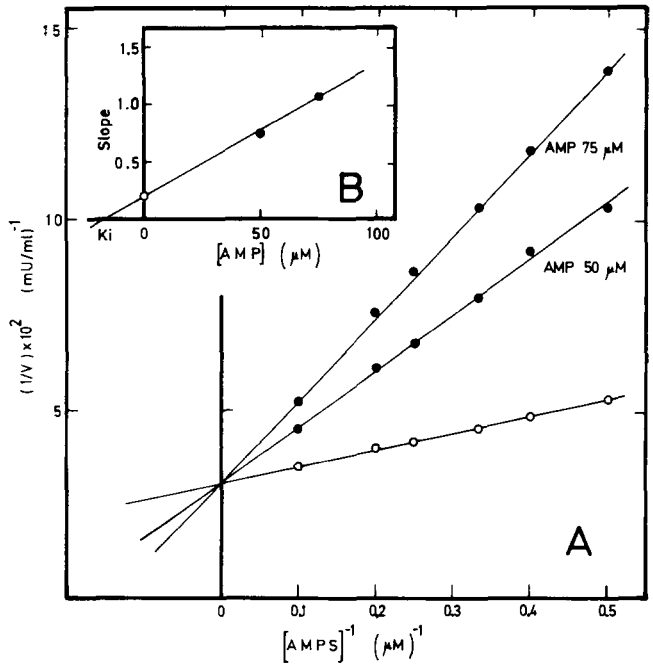

FIG. 5. **Inhibition of adenylosuccinate lyase by AMP.** The enzyme preparation used was from step 4 of purification. Where indicated, the reaction mixture was supplemented with AMP. The results are presented in a double reciprocal plot (*A*) and replot of slopes *versus* AMP concentration (*B*).

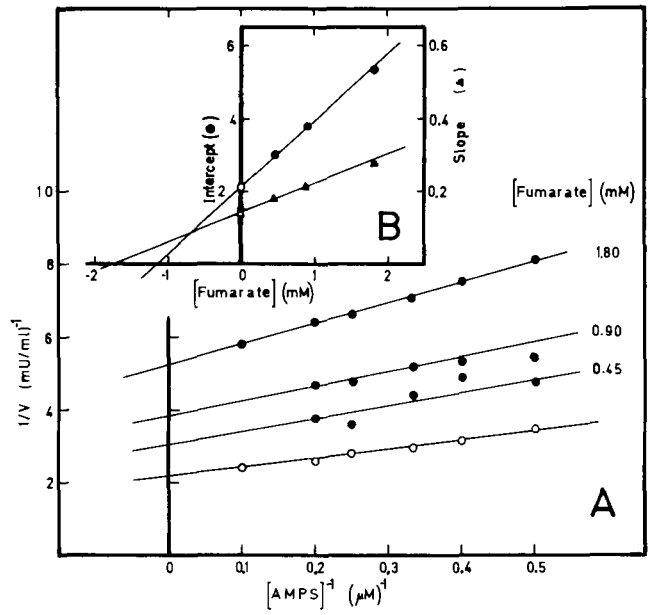

FIG. 6. **Inhibition of adenylosuccinate lyase by fumarate.** The enzyme preparation was from step 4 of purification. Where indicated, the reaction mixture was supplemented with fumarate. The results are presented in a double reciprocal plot (*A*) and as replots of slopes *versus* fumarate concentration (*B*).

Polybuffer 74 used in the previous wash at a flow rate of 24 ml/h. Fractions of 1.6 ml were collected, and the pH value was determined shortly after being eluted. When the pH was below 6.5, 30 μl of 1 M Tris (base) were added immediately to the fraction to bring the pH value to around 7–7.5, to protect the enzyme from inactivation.

The enzyme eluted as a symmetric peak corresponding to a pI of 5.04. About 17% of the activity applied to the column was recovered in that peak.

DISCUSSION

As shown above, adenylosuccinate lyase from *Artemia* cysts may adopt different molecular forms depending on the pH of the medium. Forms of 200 kDa and larger than 500 kDa were detected after chromatography on Sepharose CL-6B using elution buffers of pH 8.7 and 6.5, respectively. The existence of other forms could be observed at intermediate pH values. Similar findings were obtained with sucrose gradient centrifugation. The possible interconversion among the different forms of adenylosuccinate lyase was studied by subjecting the enzyme to two chromatographic runs on Sepharose CL-6B at two different pH values (Fig. 2). The 200-kDa form obtained by chromatography at pH 8.7 did not revert to a larger molecular size upon re-chromatography at pH 6.6 (Fig. 2*A*); the higher molecular mass form obtained at pH 7.1 passed to a 200-kDa form after re-chromatography at pH 8.7 (Fig. 2*B*); from the two peaks of activity obtained at pH 7.5, only one, the smallest one (200 kDa), was recovered after re-chromatography at the same pH (Fig. 2*C*). It is apparent that under our experimental conditions, only the 200-kDa form was obtained in the second chromatographic run regardless of the pH of the elution buffers used in the first and second chromatography. This could mean, among other possibilities, that the 200-kDa form is the more stable form of the enzyme or that upon chromatography on Sepharose CL-6B, a factor is lost which is essential for the conversion of the 200-kDa form to higher molecular forms. Such conversion is possible in the 27,000 × *g* supernatants. In supernatants obtained at pH 8.7, in which only the 200-kDa form was detected, as shown by sucrose gradient centrifugation, the larger form of the enzyme appears after lowering the pH to 6.8.

The enzyme was purified to near homogeneity from *Artemia* cysts. It had a molecular mass of 200 kDa and a single subunit of 56 kDa as revealed by electrophoresis on SDS-discontinuous, polyacrylamide slab gel. The 200-kDa form could represent a tetramer, and larger molecular forms could be due to association of monomers with or without participation of a factor essential for the association but not for the catalytic activity. We have not detected the occurrence of 150-, or 100- or 55-kDa forms in native conditions, because they are either unstable or inactive.

The results presented above can be discussed in relation to previous findings on adenylosuccinate lyase from other sources. Woodward and Braymer (16) purified to homogeneity the enzyme from *Neurospora* and studied its molecular properties by different methods, including sedimentation equilibrium and sucrose gradient centrifugation. An unique active form of 200 kDa and other inactive forms of 55 and 27 kDa were found. They determined the molecular weight of the enzyme, either purified to homogeneity or from crude extracts, by sucrose gradient centrifugation at pH 8.0. Their analytical ultracentrifuge studies were carried out at pH 7.1 and 5.2, but using a purified enzyme preparation which had been obtained after several steps at pH 8.0. Under their experimental conditions and according to our results (see Fig. 2), the possible existence of molecular forms larger than 200 kDa could have escaped them. However, the possibility that adenylosuccinate lyases from *Artemia* and *Neurospora* behave differently can not be disregarded. In other publications not specifically centered on the molecular structure of adenylosuccinate lyase and using other biological systems, M_r = 230,000 (human erythrocytes (17)) and 300,000 (*Leishmania donovani* (18)) have been reported for this enzyme.

During the development from gastrula to free swimming larva, *Artemia* uses stored diguanosine tetraphosphate as a source of adenine and guanine nucleotides (1), although the *de novo* synthesis of purine nucleotides also takes place to some extent during this time (19). This paper widens the knowledge of the enzymatic machinery needed to transform part of the diguanosine tetraphosphate moiety into AMP. The enzymes involved in this pathway and present in *Artemia* are dinucleoside tetraphosphatase (2), GMP reductase (3), adenylosuccinate synthetase (4), and adenylosuccinate lyase (this paper). Their V_{max} values (units/g) are 0.08, 0.05, 0.05, and 0.180, and the K_m values towards their respective substrates are 5 , 5, 30, and 1.2 μM, respectively. Although the limiting step of this pathway is not known, the two first enzymes of this sequence are strongly regulated. Dinucleoside tetraphosphatase is inhibited by nucleoside 5′-tetraphosphates (K_i values in the nanomolar range), and GMP reductase is inhibited by XMP and activated by diguanosine tetraphosphate (K_a = 30 nM). The activation of this enzyme by diguanosine tetraphosphate seems to ensure that, when the concentration of diguanosine tetraphosphate is high, as happens during development from gastrula to free swimming larva, the metabolism of this nucleotide is directed towards IMP as a previous step to its transformation to adenine nucleotides. Van Denbos and Finamore (20) presented data in favor of an additional pathway in *Artemia* for the conversion of diguanosine tetraphosphate to ATP via a postulated intermediate P^1,P^4-(5′,5′-(guanosyl, adenosyl))tetraphosphate.

Finally, it is interesting to note that adenylosuccinate lyase catalyzes the elimination of fumarate from both adenylosuccinate and 5-amino-4-imidazole *N*-succinocarboxamide ribonucleotide (14, 16), the last compound being an intermediate of the *de novo* pathway of purine nucleotides. In some systems such as rat liver and spleen, changes in the level of adenylosuccinate lyase could control both the *de novo* pathway and the synthesis of AMP from IMP (21). Although it would be of interest, very little is known on the level of the enzymes of the *de novo* pathway of purine nucleotides at different stages of *Artemia* development. The knowledge of this point could throw light on the problem of the peculiar nucleotide metabolism of this crustacean.

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